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Short communication

Pre-treatment of cattle sperm and/or oocyte with antibody to lipocalin type prostaglandin D synthase inhibits *in vitro* fertilization and increases sperm–oocyte binding

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Abstract

The present study was conducted to determine the affect of pre-treating of oocytes and/or sperm with a rabbit polyclonal antibody against recombinant cattle lipocalin type prostaglandin D synthase (α L-PGDS) on *in vitro* sperm–oocyte binding and fertilization. *In vitro* matured cattle oocytes were incubated (39 °C, 5% CO_2 in air) for 1 h in the following treatments either 500 μL of fertilization medium (FM) or FM with α L-PGDS (1:2000). Frozen–thawed spermatozoa were washed by a 45/90% layered Percoll gradient centrifugation and incubated for 1 h either FM or FM with α L-PGDS. This study utilized five different treatments: (1) no antibody (control); (2) a rabbit IgG against a non-bovine antigen, bacterial histidase (α-hist); (3) α L-PGDS at fertilization time (with fertilization medium); (4) α L-PGDS-treated oocytes; or (5) α L-PGDS-treated sperm. Pre-treated oocytes were incubated with 10×10^4 washed spermatozoa per 25 oocytes. Oocytes used to assess sperm binding were stained with Hoescht 33342, and the number of sperm bound per zonae pellucidae counted. The remaining oocytes were fixed in acid alcohol, stained with 1% acetate-orcein and observed to determine the presence of pronuclei. More sperm bound to the zonae pellucidae when oocytes and/or sperm were pre-treated with α L-PGDS: (1) 26.4 ± 3.0; (2) 25.6 ± 3.0; (3) 59.7 ± 3.0; (4) 56.4 ± 3.0; and (5) 57.1 ± 3.0. Addition of α L-PGDS with sperm, oocytes, or both, decreased fertilization ($P < 0.05$) compared with the control: (1) 89.2 ± 2.0%; (2) 87.5 ± 2.0%; (3) 19.4 ± 2.0%; (4) 27.2 ± 3.1%; and (5) 14.1 ± 3.4%. The α L-PGDS reacts with both oocytes and spermatozoa, resulting in

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increases of *in vitro* sperm–oocyte binding and inhibition of fertilization. These observations suggest that L-PGDS may have a role in cattle fertilization.
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Keywords: Lipocalin type prostaglandin D synthase (L-PGDS); Cattle; Sperm–oocyte binding; Fertilization

1. Introduction

Lipocalin type prostaglandin D synthase (L-PGDS) was first described as a major protein of cerebrospinal fluid and called β-trace (Clausen, 1961). Nagata et al. (1991) determined that L-PGDS catalyzed the isomerization of prostaglandin (PG) H^2 to PGD_2, the major prostanoid produced in the central nervous system of mammals. L-PGDS is the prime enzyme responsible for this conversion in the central nervous system (Fujimore and Urade, 2007). Other studies have showed the presence of L-PGDS in human and mouse heart (Urade et al., 1999; Otsuki et al., 2003), mouse liver (Fujimore et al., 2007), rat kidney (Ogawa et al., 2006), hamster ovary (Manya et al., 2000), bull epididymis and prostate gland (Rodriguez et al., 2000).

L-PGDS was also identified in bull testis, on ejaculated sperm, and as fertility-associated protein in seminal plasma (Gerena et al., 1998), but biological role of L-PGDS in male reproduction is unknown. Recently we detected L-PGDS in the cow ampullary uterine tube fluid, which may associate with zonae pellucidae (Gonçalves et al., 2008). Rabbit polyclonal antibody against recombinant cattle L-PGDS reacted with cattle oocytes incubated with uterine tube fluid and that following *in vitro* fertilization was decreased.

Although L-PGDS is present in uterine tube fluid, the role that this molecule may play in sperm–oocyte binding and fertilization is unclear. The present study was undertaken to determine if *in vitro* binding to zonae pellucidae and fertilization were affected by pre-treating sperm and/or oocytes with L-PGDS antibody.

2. Materials and methods

2.1. Pre-treatment of oocytes

Cattle ovaries of unknown breed were harvested from a local slaughter house, and cumulus–oocyte complexes (COC) were obtained from visible follicles by aspiration. The COC with uniform cytoplasm and two or more intact cumulus cells layers were incubated in medium TCM199 (Gibco™) containing 10% fetal calf serum (Gibco™), LH (0.01 units/mL), FSH (0.01 units/mL) (Sioux Biochemical, Sioux Center, IA, USA), and penicillin (100 units/mL)/streptomycin (100 μg/mL) (Gibco™) for 24 h at 39 °C in 5% CO_2 in air (Hasler et al., 1995).

In vitro matured oocytes were incubated for 1 h in 500 μL of fertilization medium (FM; Bavister et al., 1983) at 39 °C and 5% CO_2 in air with: (a) no antibody, (b) a rabbit polyclonal antibody against recombinant cattle L-PGDS (α L-PGDS; 1:2000, Gerena et al., 1998).

2.2. Pre-treatment of sperm

The present study used frozen semen from one mature Nelore bull (*Bos Indicus*). Frozen–thawed spermatozoa were washed by a 45/90% layered Percoll (Sigma®, St. Louis, MO,

USA) gradient centrifugation as described (Gonçalves et al., 2007). Pelleted spermatozoa were recovered, assessed for motility, and incubated (5×10^7 mL), as described above, for 1 h in 500 μL of fertilization medium with: (a) no antibody, or (b) α L-PGDS. After incubation, spermatozoa were separated from their incubation medium using two washes for 5 min at $500 \times g$. The first wash used 2 mL of modified Tyrode's medium (MTM; Parrish et al., 1988), and the second used 2 mL of fertilization medium.

2.3. *In vitro* sperm–oocyte binding

For studies involving sperm–oocyte binding and *in vitro* fertilization, there were five different treatments: (1) no antibody (control), (2) a rabbit IgG against a non-bovine antigen, bacterial histidase (1:2000; α-hist) (King et al., 1994), (3) α L-PGDS at fertilization time (with fertilization medium), (4) α L-PGDS-treated oocytes, or (5) α L-PGDS-treated sperm.

In vitro matured oocytes were vortexed for 2 min to remove cumulus cells, washed twice in low-bicarbonate HEPES medium (Bavister et al., 1983), and placed (25/well) in 4-well culture dishes (Nunc™; Fisher Scientific, Pittsburgh, PA, USA) containing 500 μL fertilization medium. Oocytes were co-incubated with 10×10^4 spermatozoa in the fertilization medium supplemented with 2 μg/mL of heparin (Sigma®) and 20 μL of PHE solution (20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine; Sigma®; Hasler et al., 1995). After 18 h (39 °C, 5% CO_2 in air), oocytes designated for evaluation of sperm–oocyte binding were washed once in HEPES and placed, 10 per slide, under a coverslip mounted with paraffin wax petroleum jelly at each corner. The coverslip was lowered over the oocytes until they burst, and the cytoplasm was rinsed away with HEPES. The zonae pellucidae and any spermatozoa bound to them were stained with Hoechst fluorescent dye 33342 (Sigma®). The number of spermatozoa bound to each ZP was determined using fluorescence microscopy (Way et al., 1997).

2.4. *In vitro fertilization*

In vitro-matured oocytes were washed and incubated with 10×10^4 spermatozoa as described above. After 18 h, oocytes were vortexed, and washed twice in HEPES medium. Oocytes were placed 10 per slide under a coverslip mounted at the corners with paraffin wax and petroleum jelly. The coverslip was gently lowered over the oocytes and adhered to the slide with rubber cement. Oocytes were fixed in 3.7% paraformaldehyde and 10% Triton X-100 for 1 h, washed and transferred to a solution with PBS, 0.3% BSA, and 1% Triton for 1 h. The oocytes were stained with Hoechst 33342, and observed in the presence of two pronuclei in the cytoplasm (normal fertilization).

2.5. *Statistical analyses*

Each experiment was repeated four times and data from each experiment were pooled. Approximately 50 oocytes per treatment for sperm–oocyte binding, 90 oocytes for fertilization were evaluated in each replicate. Analysis of variance using a general linear model was performed using mean number of spermatozoa bound per zonae pellucidae for each treatment in the sperm–oocyte binding experiments, and a weighted mean based on the number of oocytes per treatment in the fertilization *in vitro* experiments. Least squares mean comparisons were used to assess sperm binding and weighted least squares mean values were used to analyze fertilization data. The significance level for all tests was $P < 0.05$.

Table 1
Mean number of spermatozoa bound per zonae pellucidae ± S.E.M. and mean percentage ± S.E.M. of oocytes fertilized the following treatments: (1) no antibody (control), (2) a rabbit IgG against a non-bovine antigen, bacterial histidase (α-hist), (3) a rabbit polyclonal antibody against recombinant bovine L-PGDS (α L-PGDS) at fertilization time, (4) α L-PGDS-treated oocytes, or (5) α L-PGDS-treated sperm

Variables	Treatments				
	1	2	3	4	5
Sperm–oocyte binding (no. of sperm/oocyte)[a]	26.4 ± 3.0 a	25.6 ± 3.0 a	59.7 ± 3.0 b	56.4 ± 3.0 b	57.1 ± 3.0 b
Fertilization rate (%)[b]	89.2 ± 2.0 a	87.5 ± 2.0 a	19.4 ± 2.0 b	27.2 ± 3.1 b	14.1 ± 3.4 b

Mean values with different letters differ ($P < 0.05$).
[a] Number bound oocyte is calculated from the number of sperm bound firmly on the oocyte.
[b] Fertilization rate was calculated from the number of oocytes co-incubated with sperm.

3. Results

Addition of a rabbit polyclonal IgG antibody against recombinant cattle L-PGDS (α L-PGDS) with sperm or/and oocytes increased sperm–oocyte binding compared to the *in vitro*-fertilized control ($P < 0.05$; Table 1). When evaluating *in vitro* fertilization, less matured oocytes were fertilized when sperm and/or oocytes were incubated with α L-PGDS ($P < 0.05$; Table 1). The pre-incubation with rabbit IgG prepared against a non-bovine antigen was performed to assess the effect of non-specific IgG. There were no significant differences between the control and medium with α-hist, suggesting that rabbit IgG alone does not negatively influence sperm binding and fertilization.

4. Discussion

Secretions from uterine tube are thought to play an important role in reproduction. There is recent evidence that L-PGDS is present in the cow ampullary uterine tube fluid suggesting a specific role in the female reproductive tract (Gonçalves et al., 2008). L-PGDS is a member of the family of transport proteins known as lipocalins, which are involved in binding lipophilic ligands such as retinoids and steroids. It also catalyzes the isomerization of PGH_2 to PGD_2, and PGD_2 is involved in many physiological activities including sleep induction, regulation of body temperature and smooth muscle contraction and relaxation (Urade et al., 1995). It is possible that L-PGDS is functioning in one or both of these roles in the female tract.

In the present study, L-PGDS antibody increased sperm–oocyte binding suggesting an important function on polyspermy. This effect on polyspermy has not been previously reported, but it was know that L-PGDS does affect reproductive functions. Pre-treating oocytes and/or sperm with L-PGDS antibody reduced *in vitro* fertilization. The biological role of L-PGDS in the female reproductive tract is beginning to be explored. Retinoids are required for normal development and maintenance of many body tissues, including epithelia (Skinner, 1991). Transport of retinoids through the body is facilitated by binding proteins and L-PGDS binds retinoic acid and retinal with high affinity (Urade et al., 1996). It is suspected that L-PGDS serves as a retinoid transporter within the male reproductive tract, carrying retinoids required for normal testicular function and maintenance of spermatogenesis (Skinner, 1991). A possible function within the uterine tube is to moderate the concentration of retinoids. Additionally, smooth muscle contraction is involved in the transport of gametes and embryos through the uterine tube. However, the role of L-PDGS

in the production of PGD_2 in the uterine tube is purely speculative because its enzymatic activity within the uterine tube has not been demonstrated.

In conclusion, the present studies have demonstrated that pre-treating oocytes and/or sperm with a rabbit polyclonal antibody against recombinant cattle L-PGDS increased sperm–oocyte binding, and inhibited *in vitro* fertilization. However, more studies are necessary to better understand the role and L-PGDS enzymatic activity in the bovine uterine tube, and fertilization.

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